Cellular Signalling 20 (2008) 1865–1872

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Molecular insights into connective tissue growth factor action in rat pancreatic stellate cells

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ARTICLE INFO

Article history:
Received 7 May 2008
Received in revised form 25 June 2008
Accepted 27 June 2008
Available online 2 July 2008

Keywords:
Connective tissue growth factor
Interleukin-6
Pancreatic stellate cells

ABSTRACT

Pancreatic fibrosis, a key feature of chronic pancreatitis and pancreatic cancer, is mediated by activated pancreatic stellate cells (PSC). Connective tissue growth factor (CTGF) has been suggested to play a major role in fibrogenesis by enhancing PSC activation after binding to $\alpha 5\beta 1$ integrin. Here, we have focussed on molecular determinants of CTGF action. Inhibition of CTGF expression in PSC by siRNA was associated with decreased proliferation, while application of exogenous CTGF stimulated both cell growth and collagen synthesis. Real-time PCR studies revealed that CTGF target genes in PSC not only include mediators of matrix remodelling but also the proinflammatory cytokines interleukin (IL)-1β and IL-6. CTGF stimulated binding of NF-κB to the IL-6 promoter, and siRNA targeting the NF-κB subunit RelA interfered with CTGF-induced IL-6 expression, implicating the NF-κB pathway in the mediation of the CTGF effect. In further studies, we have analyzed regulation of CTGF expression in PSC. Transforming growth factor-β1, activin A and tumor necrosis factor-α enhanced expression of the CTGF gene, while interferon-γ displayed the opposite effect. The region from −74 to −125 of the CTGF promoter was revealed to be critical for its activity in PSC as well as for the inhibitory effect of interferon-γ. Taken together, our results indicate a tight control of CTGF expression in PSC at the transcriptional level. CTGF promotes fibrogenesis both directly by enhancing PSC proliferation and matrix protein synthesis, and indirectly through the release of proinflammatory cytokines that may accelerate the process of chronic inflammation.

1. Introduction

The CCN family of secreted proteins comprises six structurally related members that specifically associate with the extracellular matrix (ECM). CCN proteins act as regulators of diverse cellular functions, including mitogenesis, apoptosis, ECM production, adhesion and migration, as well as complex biological processes such as angiogenesis, chondrogenesis and osteogenesis [1,2]. As a major principle of CCN protein action, modification of signal transduction pathways induced by other molecules (which vary depending on the particular biological system) has emerged. Recent studies have linked the CCN family member connective tissue growth factor (CTGF; also known as CCN2) to the induction of fibrogenesis and, under pathological conditions, the development of fibroproliferative diseases [3]. At least in part, CTGF exerts its profibrotic effects in direct synergy with transforming growth factor-beta (TGF-β), e.g. by mediating downstream actions of this cytokine. Furthermore, TGF-β is a well-established inductor of CTGF gene expression [3].

In the pancreas, pancreatic stellate cells (PSC) have been identified both as a source of CTGF and a target of CTGF action [4,5]. PSC are the main ECM protein-synthesizing cells in the organ [6,7]. The cells have recently been shown to display a transcriptional phenotype that closely resembles the one of hepatic stellate cells, the principle effector cells in liver fibrosis [8]. Dysregulated production of ECM by activated PSC has been implicated in the progression of chronic pancreatitis and pancreatic cancer (PC); two diseases that are characterized by an extended organ fibrosis [9,10]. In detail, CTGF has been shown to enhance collagen synthesis and proliferation of PSC, and to promote both adhesion and migration of the cells [4]. CTGF effects on PSC are mediated, at least in part, by the cell surface integrin $\alpha 5\beta 1$ and heparan sulfate proteoglycan receptors [4,5]. With respect to PC, it is important to note that CTGF has also been suggested to promote cancer growth directly by stimulating proliferation and migration of the tumor cells, while antibodies to CTGF exert antiproliferative, antiangiogenetic and antimetastatic effects [11,12].

Abbreviations: ECM, extracellular matrix; CTGF, connective tissue growth factor; TGF-β, transforming growth factor-beta; PSC, pancreatic stellate cell; PC, pancreatic cancer; PDGF, platelet-derived growth factor; ERK, extracellular signal-regulated kinase; STAT, signal transducer and activator of transcription; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; ECL, enhanced chemoluminescence; IFN, interferon; BrdU, 5-bromo-2′-deoxyuridine; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; HPRT, hypoxanthine-guanine phosphoribosyl transferase; NF, nuclear factor; SEM, standard error of the mean; TNF, tumor necrosis factor.

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doi:10.1016/j.cellsig.2008.06.016

The purpose of this study was to gain further insights into the molecular determinants of CTGF action in PSC. Therefore, we took advantage of an established cell culture model that has been used in the past to characterize both extra- and intracellular mediators of PSC activation; a process characterized by cell proliferation and the exhibition of a myofibroblastic PSC phenotype with an enhanced capacity of ECM synthesis [6,7,13]. Thus, it has previously been shown that TGF-β1 and platelet-derived growth factor (PDGF) are potent inductors of ECM synthesis and PSC proliferation, respectively [13–15]. In our studies, we have focussed on mediators and targets of CTGF signalling in PSC as well as on the regulation of CTGF expression. It was revealed that CTGF contributes to PSC proliferation through an autocrine loop. Intracellular mediators of the CTGF signal involve extracellular signal-regulated kinases (ERK) 1 and 2 as well as signal transducer and activator of transcription (STAT) 3. CTGF promoter activity in PSC essentially depends on the integrity of the region from −74 to −125 of the CTGF gene. Interestingly, CTGF target genes in PSC include the proinflammatory cytokines interleukin (IL)-1β and IL-6, suggesting a previously unrecognized direct role of CTGF in pancreatic inflammation.

2. Materials and methods

2.1. Material

Iscove's modified Dulbecco's medium (IMDM) and supplements for cell culture were obtained from Biochrom (Berlin, Germany). Nycodenz from Nycomed (Oslo, Norway) and the Topo TA Cloning kit as well as TRIzol reagent from Invitrogen (Karlsruhe, Germany). Radiochemicals, the enhanced chemoluminescence (ECL) Plus kit and horseradish-peroxidase labelled antibodies were purchased from GE Healthcare (Freiburg, Germany), and all other antibodies from New England BioLabs (Frankfurt, Germany). Reverse transcription and Taqman™ reagents were delivered by Applied Biosystems (Foster City, CA, USA), the plasmids PGL3-Basic as well as pGL 4.70 by Promega (Madison, WI, USA), human CTGF by EMP Genetech (Ingolstadt, Germany) and recombinant cytokines (rat interferon [IFN]-γ, activin A and PDGF, human TGF-β1) by R&D Systems (Minneapolis, MN, USA). DNA oligonucleotides were synthesized by BioTeZ (Berlin, Germany). The 5-bromo-2′-deoxyuridine (BrdU) labelling and detection enzyme-linked immunosorbent assay kit, polynucleotide kinase and FuGene™ were from Roche Diagnostics (Mannheim, Germany), and collagenase as well as standard laboratory chemicals from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Pancreatic stellate cells were isolated from male LEW.1W inbred rats by collagenase digestion of the pancreas followed by Nycodenz density gradient centrifugation as previously described [13]. Isolated PSC were cultured in IMDM supplemented with 17% fetal calf serum, 1% non-essential amino acids (dilution of a 100 X stock solution), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. All experiments were performed with cells growing in primary culture, or, depending on the experimental settings, with cells of the first passage. If replating of the cells was required, PSCs were harvested by trypzination on day seven after isolation and recultured at equal seeding densities.

2.3. Cell proliferation assay

PSC were cultured in 96-well plates in serum-free culture medium containing supplements as indicated. After 24 h, BrdU labelling was initiated by adding labelling solution at a final concentration of 10 μM. Another 24 h later, labelling was stopped, and BrdU uptake was measured according to the manufacturer's instructions.

2.4. Inhibition of gene expression using siRNA

siRNAs were purchased from Qiagen (Hilden, Germany) and applied at the indicated concentrations according to the manufacturer's instructions, using *HiPerFect* transfection reagent (Qiagen). The following siRNAs, targeting CTGF or p65RelA, were included into the investigations: Rn_CTGF_3_HP, Rn_CTGF_6_HP, Rn_RelA_1_HP, and Rn_RelA_2_HP. Efficiency of siRNA treatment was monitored by analyzing CTGF and p65 RelA mRNA levels as indicated. To study the effects of CTGF siRNA on PSC growth, cells pretreated with siRNA for 66 h were labelled with BrdU for another 6 h before DNA synthesis was quantified by determination of BrdU incorporation. In all experiments, a non-silencing siRNA oligonucleotide was included as a negative control.

2.5. Quantification of collagen synthesis

Collagen synthesis was assessed through the quantification of [^{3}H]-proline incorporation into acetic acid-soluble proteins as previously described [16]. Treatment of PSC with CTGF was for 48 h. Raw data of [^{3}H]-proline incorporation were normalized on the basis of absolute cell counts determined by trypan blue staining of PSC cultured in parallel under identical conditions, except that no [^{3}H]-proline was added.

2.6. Immunoblotting

Protein extracts of stellate cells were prepared, adjusted to identical protein concentrations and subjected to immunoblotting as previously described [13]. The blots were developed with ECL Plus and analyzed using a Kodak Image Station (440 CF; Kodak 1D image analysis software, version 3.5).

2.7. IL-6 ELISA

Quantitative determinations of IL-6 protein levels were performed using a rat IL-6-specific ELISA (BioSource International, Camarillo, CA, USA). Therefore, PSC were pretreated by siRNA incubation, serum withdrawal and CTGF stimulation as indicated. Cell culture supernatants were collected and stored at −80 °C until use. The ELISA was performed according to the manufacturer's instructions.

2.8. Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

Total RNA from cells pretreated as indicated was isolated with TRIzol reagent according to the manufacturer's instructions. Next, RNA was reverse transcribed into cDNA by means of TaqMan™ Reverse Transcription Reagents and random hexamer priming. Target cDNA levels were analyzed by quantitative real-time PCR using TaqMan™ Universal PCR Master Mix and the following Assay-on-Demand™ rat gene-specific fluorescently labelled TaqMan™ MGB probes in an ABI Prism 7000 sequence detection system (Applied Biosystems): Rn00573960_g1 (CTGF), Rn00580432_m1 (IL-1β), Rn 00561420_m1 (IL-6), Rn01538167_m1 (matrix metalloproteinase [MMP]-2), Rn00579162_m1 (MMP-9), Rn00572010_m1 (TGF-β1), Rn00587558_m1 (tissue inhibitor of matrix metalloproteinase [TIMP]-1), Rn00801649_g1 (procollagen, type 1, alpha 1), Rn01502266_m1 (RelA) and Rn01527838_g1 (hypoxanthine-guanine phosphoribosyl transferase [HPRT]; house-keeping gene control). PCR conditions were as follows: 95 °C for 10 min, 55 cycles of 15 s at 95 °C/1 min at 60 °C. PCR reactions were performed in triplicate. The relative expression of each mRNA compared with HPRT was calculated according to the equation $\Delta Ct = Ct_{target} - Ct_{HPRT}$. The relative amount of target mRNA in control cells and cells treated as indicated was expressed as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct_{\text{treatment}} = Ct_{\text{treatment}} - \Delta Ct_{\text{control}}$.

2.9. Analysis of CTGF promoter activity

A 780 bp fragment of the human CTGF promoter (GenBank Accession Number AF316368, positions −740 to +40 with the transcription initiation site as +1) was amplified by PCR using genomic DNA isolated from mononuclear blood cells (see Table 1 for primer sequences). The resulting PCR product was ligated into pCR 2.1-Topo using the Topo TA Cloning kit and verified by dideoxy sequencing. Subsequently, the fragment was subcloned into the plasmid PGL3-Basic upstream of the firefly luciferase reporter gene, generating the reporter vector PGL3-CTGFΔ780. In an analogous manner, a series of shorter CTGF promoter fragments was generated and cloned (Table 1).

CTGF promoter activity was analyzed in transient transfection assays, using the Dual-Luciferase reporter assay system (Promega). In this system, the activities of the experimental firefly luciferase and a constitutively expressed renilla luciferase (encoded by a co-transfected plasmid) are measured sequentially from a single sample to allow normalisation of transfection efficiencies. Therefore, subconfluent cultures of PSC growing in 96-well plates were transfected with 200 ng of the indicated PGL3B-vector and 10 ng of the renilla luciferase vector pGL 4.70 per well, using *FuGene™ HD Transfection Reagent* according to the manufacturer's instructions. In some studies, transfected cells were treated with IFN-γ for 24 h as indicated. Luciferase activities in the attached cells were measured as suggested by the vendor, and ratios of firefly and renilla luciferase activities were determined.

2.10. Electrophoretic mobility shift assays (EMSA)

PSC growing in 6-well plates were incubated for 12 h in FCS-free culture medium, before they were stimulated with CTGF as indicated. Nuclear extracts were prepared as previously described [13,17]. For EMSA experiments, nuclear proteins of 1×10^5 cells were incubated with double-stranded oligonucleotides which were end-labelled with [γ-^{32}P] ATP by polynucleotide kinase. The sequence of the nuclear factor (NF)-κB binding probe derived from the IL-6 promoter was 5′-*ATG*1*GGGATTTTCCCATGAGC*3′ (consensus binding motif italic) [18]. Binding reaction and supershift analysis (initiated by adding 0.5 μg antibody) were performed as described [13,17]. Protein-DNA complexes were analyzed by electrophoretic separation on a 6% non-denaturating polyacrylamide gel. Dried gels were exposed to X-ray film.

2.11. Statistical analysis

Results are expressed as means ± standard error of the mean (SEM) for the indicated number of independent cultures per experimental protocol. Unless indicated otherwise, statistical significance was checked using Wilcoxon's rank sum test. $p<0.05$ was considered to be statistically significant.

3. Results

3.1. Biological effects of CTGF in PSC and mediation of the CTGF signal

Initial experiments indicated that exogenously applied CTGF stimulated PSC proliferation and collagen synthesis with a maximum effect similar to the one of the strong mitogen PDGF and the best-established stimulator of ECM synthesis, TGF-β1 (Fig. 1). Because PSC themselves express CTGF [19], we also studied how inhibition of CTGF synthesis affects PSC growth. Application of CTGF siRNA efficiently reduced CTGF mRNA levels in PSC, and simultaneously significantly diminished DNA synthesis of the cells (Fig. 2). To further characterize responsiveness of PSC to CTGF, activation of intracellular signal transduction pathways was analyzed. Two previously identified mediators of the CTGF signal, ERK 1/2 and STAT3 [20], were found to become phosphorylated in response to CTGF treatment (Fig. 3).

Table 1
CTGF promoter fragments and primer sequences (5′–3′) used for amplification

Name of fragment	Promoter region	Upstream primer	Downstream primer
Δ780	−740–+40	GCCACTCCTCCCTTGTCCTTGC	CACTGGCTGTCTCCTCTC
Δ595	−555–+40	CAGGTAGGCATCTTGAGG	
Δ396	−356–+40	GGACAGAACAGCGCAAAC	
Δ281	−241–+40	GCTGCTCGAAATACTGCGC	
Δ165	−125–+40	GTGAGTTGATGAGGCAGG	
Δ114	−74–+40	TGTGTACACTCCATTCAGC	

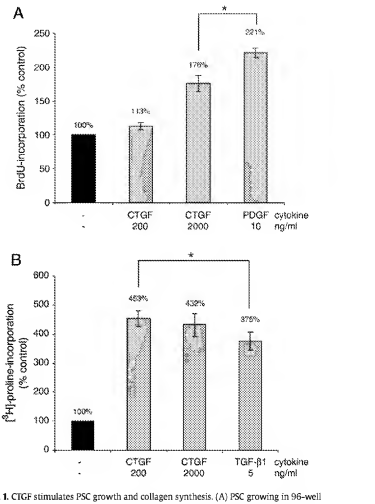

Fig. 1. CTGF stimulates PSC growth and collagen synthesis. (A) PSC growing in 96-well plates were incubated under serum-free conditions with CTGF and PDGF at the indicated concentrations for 24 h. Subsequently, cells were labelled with BrdU for 24 h, and proliferation assessed with the BrdU DNA-incorporation assay. One hundred percent BrdU incorporation corresponds to untreated PSC. Data from 6 separate cultures were used to calculate mean values and SEM. * $P<0.05$ versus untreated control cultures. (B) PSC growing in 12-well plates in complete culture medium were stimulated with CTGF and TGF-β1 at the indicated concentrations for 48 h. Collagen synthesis was analyzed through the quantification of [^{3}H]-proline incorporation into acetic acid-soluble proteins, and raw data were normalized for differences in cell growth rates as described under Materials and methods. One hundred percent [^{3}H]-proline incorporation corresponds to untreated PSC. Data are expressed as mean (±SEM) of 6 independent experiments. * $P<0.05$ versus untreated control cultures.

3.2. CTGF target genes in PSC

In our investigations of CTGF target genes, we initially focussed on mediators of matrix remodelling known to be expressed by PSC. As shown in Fig. 4A, CTGF rapidly and strongly up-regulated expression of MMP-9. Simultaneously, it induced smaller, but also significant increases of the mRNA levels of the MMP inhibitor TIMP-1 (B) as well

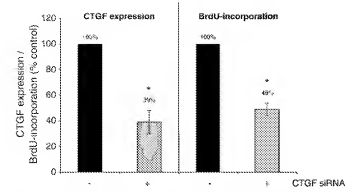

Fig. 2. CTGF siRNA inhibits PSC proliferation. Cultured PSC were transfected with CTGF siRNA (mixture of Rn_CTGF_3_HP and Rn_CTGF_6_HP; each at 5 nM) or a non-silencing control siRNA (at 10 nM) as indicated. Columns 1 and 2: After 48 h of incubation, mRNA expression of CTGF and the housekeeping gene HPRT was analyzed by real-time PCR, and relative amounts of CTGF mRNA were calculated. One hundred percent CTGF expression corresponds to PSC transfected with control siRNA. Data of 5 independent experiments (with triplicate samples) were used to calculate mean values and SEM. * $P < 0.05$ versus cultures transfected with non-silencing siRNA. Columns 3 and 4: Cell proliferation was assessed with the BrdU DNA-incorporation assay as described in the Materials and methods section. One hundred percent cell proliferation corresponds to PSC transfected with non-silencing siRNA. Data from 6 separate cultures were used to calculate mean values and SEM. * $P < 0.05$ versus cultures transfected with control siRNA.

as TGF-β1 (C) after 8 h of incubation. In contrast, expression of MMP-2 and type I collagen mRNA remained unchanged (data not shown).

Previously, PSC have also been shown to be a source of pro-inflammatory cytokines [21–23]. In studies aimed at elucidating regulation of IL-1β and IL-6 expression, we also asked for a possible role of CTGF. Strikingly, CTGF induced a rapid and massive increase of both IL-1β and IL-6 mRNA expression (Fig. 5). To elucidate the molecular mechanisms underlying induction of IL-6 expression by CTGF, EMSA experiments were performed. Using an IL-6 promoter-derived oligonucleotide with a NF-κB binding motif [18], we observed CTGF-dependent protein binding (Fig. 6). The protein complex was supershifted with an antibody against the NF-κB subunit p65RelA. To address the question if NF-κB activation plays a direct role in CTGF-induced IL-6 synthesis, PSC were treated with p65RelA siRNA. Downregulation of the p65RelA mRNA level was associated with a strong decrease of CTGF-dependent IL-6 expression (Fig. 7A). At the protein level, qualitatively identical effects were observed (Fig. 7B): CTGF stimulation induced an increase of IL-6 levels in PSC culture supernatants. When PSC were pretreated with p65RelA siRNA, the CTGF effect was significantly diminished.

3.3. Regulation of CTGF expression

In subsequent studies, we focussed on the transcriptional control of CTGF gene expression in PSC. In accordance with previous own studies using immortalized PSC lines [24], TGF-β1 was found to enhance CTGF expression whereas IFN-γ displayed the opposite effect. With activin A and tumor necrosis factor (TNF)-α, two novel inductors of CTGF gene expression in PSC were identified (Fig. 8).

To decipher regions of the CTGF promoter essential for its activity in PSC, luciferase reporter gene assays were performed. Using CTGF promoter variants of different length (Table 1), we found that the constructs Δ780, Δ595, Δ396 and Δ281 all displayed similar activities, whereas further deletions (Δ165 and Δ114 constructs) were accompanied by a significant decrease of CTGF promoter activity (Fig. 9A). We have previously reported an inhibitory effect of IFN-γ on CTGF promoter activity [24]. Interestingly, the Δ114 construct also displayed a significantly reduced IFN-γ responsiveness compared to the Δ780 fragment (Fig. 9B), whereas IFN-γ responsiveness of all other constructs did not significantly differ (data not shown).

4. Discussion

In recent years, it has been acknowledged that fibrosis not only accompanies pancreatic cancer but plays an active role in tumor progression. Thus, ECM proteins and cytokines/growth factors released by stroma cells have been shown to stimulate tumor growth and invasion, induce chemoresistance and suppress tumor cell apoptosis (reviewed in [25]). Furthermore, in chronic pancreatitis, a risk factor of PC, accumulation of ECM contributes to the development of an exocrine and endocrine insufficiency of the gland [26,27]. The main source of ECM proteins in the diseased pancreas are activated

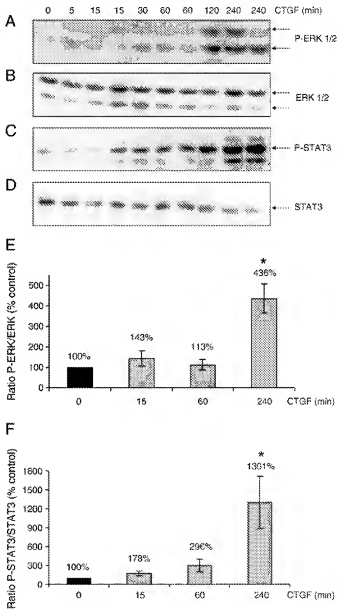

Fig. 3. CTGF induces phosphorylation of ERK 1/2 and STAT3. PSC growing in 12-well plates were starved from serum for 16 h before they were stimulated with CTGF (200 ng/ml) for the indicated periods of time. (A, C) ERK 1/2 and STAT3 phosphorylation were analyzed by immunoblotting. (B, D) Reprobing of the blots with anti-ERK 1/2 and anti-STAT3 protein-specific antibodies revealed no systematic differences of the ERK 1/2 and STAT3 amount among the samples. (E, F) Luminescence signal intensities of phospho-ERK 1/2, ERK 1/2 protein, phospho-STAT3 and STAT3 protein were quantified using Kodak 1D image analysis software. Subsequently, the ratios phospho-ERK/ERK protein and phospho-STAT3/STAT3 protein were calculated. A ratio of one hundred percent corresponds to unstimulated control cultures. Data of 6 independent experiments were used to calculate mean values and SEM. * $P < 0.05$ versus control cultures.

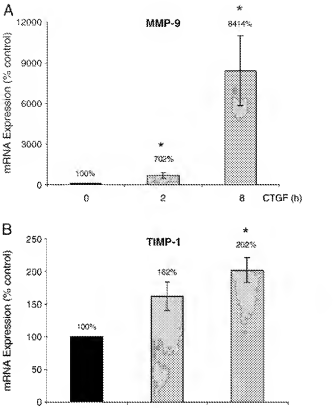

Fig. 4. Effects of CTGF on the mRNA expression of genes involved in matrix remodelling. PSC growing in 6-well plates were starved from serum for 1 h before they were stimulated with CTGF (2 μg/ml) as indicated. The mRNA expression of MMP-9 (A), TIMP-1 (B), TGF-β1 (C) and the housekeeping gene HPRT was analyzed by real-time PCR, and relative amounts of target mRNA were calculated. One hundred percent mRNA expression of each gene corresponds to untreated PSC. Data of 5 independent experiments (with triplicate samples) were used to calculate mean values and SEM. * $P<0.05$ versus control cultures.

PSC [6,7]. While activation of PSC itself is likely to be part of a normal wound healing reaction in response to pancreatic injury, perpetuation of PSC activation under persistent pathological conditions is thought to contribute to the development of fibrosis. In this process, autocrine loops of PSC-derived cytokines and growth factors play an essential role [25]. Here, we have focussed on one important mediator of PSC activation, CTGF, and studied molecular principles of its action.

In accordance with another recent study [4], our initial experiments revealed a strong stimulatory effect of CTGF on collagen synthesis by PSC. We also observed a mitogenic action of the protein (if applied to the cells) and a diminished growth rate in response to a knock-down of its expression, implicating CTGF in the maintenance of PSC proliferation.

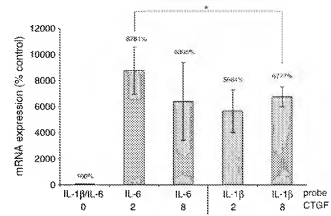

Fig. 5. Effects of CTGF on the mRNA expression of IL-1 and IL-6. PSC growing in 6-well plates were starved from serum for 1 h before they were stimulated with CTGF (2 μg/ml) for the indicated periods of time. The mRNA expression of IL-1β, IL-6 and the housekeeping gene HPRT was analyzed by real-time PCR, and relative amounts of target mRNA were calculated. One hundred percent mRNA expression of each gene corresponds to untreated PSC. Data of 5 independent experiments (with triplicate samples) were used to calculate mean values and SEM. * $P<0.05$ versus control cultures.

CTGF has been shown to exert its effects on PSC by targeting integrin α5β1 and heparan sulfate proteoglycan receptors [4,5], whereas the downstream effectors of the CTGF signal in the cells have not been fully characterized yet. Our data indicate an activation of the ERK 1/2 pathway and also of STAT3 by the protein, confirming similar observations in other cells [20]. The main novel findings of this study, with respect to CTGF action, refer to the identification of CTGF target genes in PSC.

First, CTGF not only increased mRNA levels of the profibrogenic mediators TGF-β1 and TIMP-1 (which favours ECM accumulation by blocking MMP activity [28]), but at the same time also stimulated expression of the matrix-degrading collagenase MMP-9. These data suggest that the effect of CTGF involves both an enhanced synthesis and turnover of ECM in the pancreas. It has to be stated, however, that the effects of CTGF on TGF-β1, TIMP-1 and MMP-9 mRNA expression

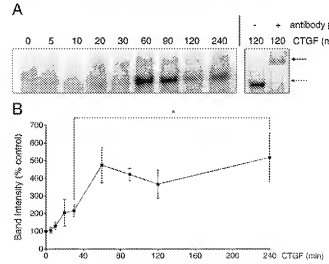

Fig. 6. CTGF activates binding of NF-κB to the IL-6 promoter. Serum-starved PSC growing in 6-well plates were incubated with CTGF (2 μg/ml) for the indicated periods of time. (A) Nuclear extracts were subjected to EMSA analysis using an IL-6 promoter-derived, [^{32}P]-labelled oligonucleotide with a NF-κB binding motif. Supershift analysis was performed by incubating the binding reaction with a p65RelA-specific antibody. Shifted and supershifted complexes are pointed out by arrows. (B) Band intensities of NF-κB/oligonucleotide complexes were further analyzed by scanning densitometry. A band intensity of one hundred percent corresponds to untreated PSCs. Data of at least 5 independent experiments were used to calculate mean values and SEM. * $P<0.05$ versus control cultures.

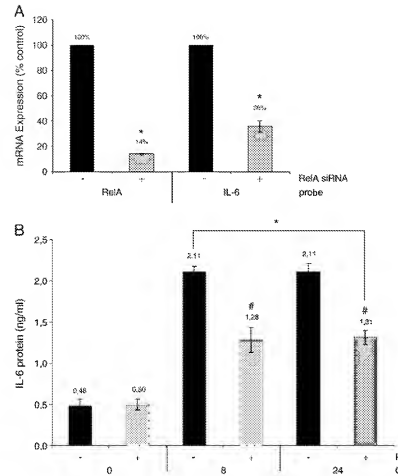

Fig. 7. RelA siRNA inhibits CTGF-induced IL-6 expression. Cultured PSC were transfected with RelA siRNA (mixture of Rn_RelA_1_HP, and Rn_RelA_2_HP; each at 2.5 nM) or a non-silencing control siRNA (at 5 nM) as indicated. After 48 h of incubation, FCS was withdrawn from the cells for 1 h before they were stimulated with CTGF (2 μg/ml) for 2 h (A), or as indicated (B). (A) Expression of RelA, IL-6 and HPRT (housekeeping gene mRNA) was analyzed by real-time PCR, and relative expression levels were calculated. One hundred percent RelA or IL-6 expression corresponds to PSC transfected with control siRNA. Data of 5 independent experiments (with triplicate samples) were used to calculate mean values and SEM. * $P<0.05$ versus cultures transfected with non-silencing siRNA. (B) IL-6 protein levels in cell culture supernatants were analyzed by ELISA. Mean values and SEM were calculated from 6 independent samples. Statistical significance was checked using a *t*-test. * $P<0.05$ versus control cultures (identical treatment with siRNA; no CTGF application). # $P<0.05$ versus cells cultured with non-silencing siRNA (identical time of CTGF stimulation).

have not been confirmed at the protein level yet. Interestingly, induction of collagen expression was observed at the protein level but not at the level of mRNA (procollagen, type 1, alpha 1).

Secondly, we observed a strong increase of IL-1β and IL-6 gene expression in CTGF-treated PSC. Both cytokines are key mediators of pro-inflammatory reactions, implicating for the first time CTGF into the enhancement of local inflammatory reactions in the pancreas. Moreover, IL-1β and IL-6 have previously been shown to act on PSC through autocrine loops that are likely to play a role in the induction and maintenance of PSC activation [22,23]. In studies aimed at elucidating molecular mechanisms of CTGF-dependent IL-6 expression, we focussed on the transcriptional control of the CTGF gene. We observed that CTGF induced binding of NF-κB to the IL-6 promoter, and that down-regulation of p65RelA protein levels significantly diminished IL-6 expression in response to CTGF stimulation. Taken together, these data suggest an essential role of NF-κB in the mediation of the CTGF signal to the IL-6 promoter.

In a previous study using an immortalized PSC line, we had observed a stimulatory effect of TGF-β1 and an inhibitory action of IFN-γ on CTGF gene expression [21]. We now confirmed these data in freshly isolated cells and identified the TGF-β family member activin A and TNF-α as two additional inductors of CTGF expression in PSC. Both mediators have previously been suggested as stimulators of the PSC activation process [10,29,30]. Interestingly, the effect of TNF-α on

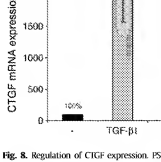

Fig. 8. Regulation of CTGF expression. PSC growing in 6-well plates were starved from serum for 1 h before they were stimulated with TGF-β1 (5 ng/ml; 8 h), IFN-γ (100 ng/ml, 24 h), TNF-α (10 ng/ml, 8 h), and activin (Act) A (100 ng/ml, 8 h) as indicated. The conditions of stimulation (time and concentration) were established for each cytokine in preliminary experiments and optimized with respect to maximum efficiency (data not shown). The mRNA expression of CTGF and the housekeeping gene HPRT was analyzed by real-time PCR, and relative amounts of target mRNA were calculated. One hundred percent CTGF-expression corresponds to untreated PSC. Data of at least 5 independent experiments (with triplicate samples) were used to calculate mean values and SEM. * $P<0.05$ versus control cultures.

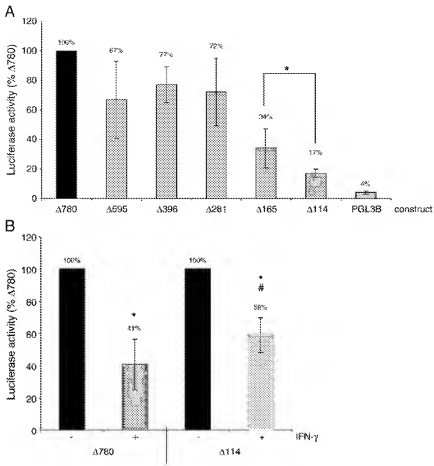

Fig. 9. Analysis of CTGF promoter activity. (A) Using *FuGene™ HD Transfection Reagent*, PSC growing in 96-well plates were transfected with pGL 4.70 as well as one of the following PGL3-derived CTGF promoter vectors: Δ780, Δ595, Δ396, Δ281, Δ165, Δ114 and PGL3-Basic (PGL3B). 24 h later, *firefly* and *renilla* luciferase activities were measured, and the ratio was determined for each sample. A ratio of one hundred percent corresponds to cells transfected with the Δ780 promoter construct. Data from 6 separate cultures were used to calculate mean values and SEM. * $P<0.05$ versus Δ780. (B) Cells transfected with Δ780 and Δ114 as described above were stimulated with IFN-γ (100 ng/ml) for 24 h as indicated. A *firefly*/*renilla* luciferase activity ratio of one hundred percent corresponds to cells that were transfected with the indicated vector and cultured without IFN-γ. * $P<0.05$ versus identically transfected cells grown without the cytokine. # $P<0.05$ versus cells transfected with Δ780 and incubated with IFN-γ.

CTGF gene expression in PSC differs from the one in some other types of cells including skin fibroblasts, where TGF-β-induced CTGF expression was repressed by the cytokine [31]. On the other hand, TGF-β and TNF-α synergistically increased CTGF synthesis in mesangial cells [32]. The molecular basis of this cell-type specific action of TNF-α remains to be elucidated.

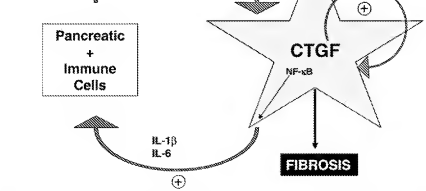

Fig. 10. Model of CTGF action in PSC. CTGF expression is induced by profibrogenic mediators such as TGF-β1 and activin A, and inhibited by IFN-γ (not shown). Through an autocrine loop, CTGF stimulates PSC proliferation, thereby promoting fibrosis. CTGF target genes in PSC include IL-1β and IL-6. The latter gene is expressed in a NF-κB-dependent manner. Both cytokines exert pro-inflammatory effects and enhance PSC activation. For details, see text.

So far, little is known about signal transduction pathways connecting IFN receptors to the CTGF promoter. Through the analysis of CTGF promoter variants, we now deciphered a relatively small region, ranging from −74 to −125, that is essential both for basal CTGF promoter activity in PSC and for its inhibition by IFN-γ. In pilot experiments, binding of distinct protein complexes to this promoter sequence (lacking established IFN response motifs) could be observed (data not shown). Further studies on the molecular coupling between IFN-γ signalling and CTGF expression may contribute to a better understanding of the antifibrotic efficiency of interferons, which is of clinical importance.

Summarized, the results of this study suggest that PSC are at multiple levels involved in pathophysiological processes associated with CTGF action. The cells are both source and target of the protein. In response to CTGF binding, autocrine and paracrine loops are activated, resulting in enhanced inflammation and fibrogenesis (Fig. 10). We propose that targeting the axis CTGF-PSC might be a promising approach in the treatment of pancreatic fibrosis.

Acknowledgements

We gratefully acknowledge the excellent technical assistance of Mrs. Helga Schulze and Mrs. Katja Bergmann. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) to R. J.

References

[1] A. Leask, D.J. Abraham, J. Cell Sci. 119 (2006) 4803.
[2] B. Perbal, Lancet 363 (2004) 62.
[3] A. Leask, D.J. Abraham, Biochem. Cell Biol. 81 (2003) 355.
[4] R. Gao, D.R. Brigstock, Gastroenterology 129 (2005) 1019.
[5] R. Gao, D.R. Brigstock, Gut 55 (2006) 856.
[6] M.G. Bachem, E. Schneider, H. Groß, H. Weidenbach, R.M. Schmid, A. Menke, M. Siech, H. Beger, A. Grünert, G. Adler, Gastroenterology 115 (1998) 421.
[7] M.V. Apte, P.S. Haber, T.L. Applegate, I.D. Norton, G.W. McCaughan, M.A. Korsten, R.C. Pirola, J.S. Wilson, Gut 43 (1998) 128.
[8] M. Buchholz, H.A. Kestler, K. Holzmann, V. Ellenrieder, W. Schneiderhan, M. Siech, G. Adler, M.G. Bachem, T.M. Gress, J. Mol. Med. 83 (2005) 795.
[9] M.G. Bachem, M. Schunemann, M. Ramadani, M. Siech, H. Beger, A. Buck, S. Zhou, A. Schmid-Kotsas, G. Adler, Gastroenterology 128 (2005) 907.
[10] P. Mews, P. Phillips, R. Fahmy, M. Korsten, R. Pirola, J. Wilson, M. Apte, Gut 50 (2002) 535.
[11] T. Aikawa, J. Gunn, S.M. Spong, S.J. Klaus, M. Korc, Mol. Cancer Ther. 5 (2006) 1108.
[12] N. Dornhofer, S. Spong, K. Bennewith, A. Salim, S. Klaus, N. Kambham, C. Wong, F. Kaper, P. Sutphin, R. Nacamuli, M. Hockel, Q. Le, M. Longaker, G. Yang, A. Koong, A. Giaccia, Cancer Res. 66 (2006) 5816.
[13] R. Jaster, G. Sparmann, J. Emmrich, S. Liebe, Gut 51 (2002) 579.
[14] T. Luttenberger, A. Schmid-Kotsas, A. Menke, M. Siech, H. Beger, A. Grünert, G. Adler, Lab. Invest. 80 (2000) 47.
[15] M.L. Kruse, P.B. Hildebrand, C. Timke, U.R. Fölsch, W.E. Schmidt, Regul. Pept. 90 (2000) 47.
[16] R. Jaster, I. Hilgendorf, B. Fitzner, P. Brock, G. Sparmann, J. Emmrich, S. Liebe, Biochem. Pharmacol. 66 (2003) 633.
[17] B. Fitzner, G. Sparmann, J. Emmrich, S. Liebe, R. Jaster, Int. J. Colorectal Dis. 19 (2004) 414.
[18] S. Plaisance, W. Vanden Berghe, E. Boone, W. Fiers, G. Haegeman, Mol. Cell. Biol. 17 (1997) 3733.
[19] T. Shinji, K. Ujike, K. Ochi, N. Kusano, T. Kikui, N. Matsumura, Y. Emori, T. Seno, N. Koide, Acta Med. Okayama 56 (2002) 211.
[20] S.H. Wu, X.H. Wu, C. Lu, L. Dong, Z.Q. Chen, Am. J. Respir. Cell Mol. Biol. 34 (2006) 65.
[21] G. Sparmann, C. Hohenadl, J. Tomoe, R. Jaster, B. Fitzner, D. Koczan, H.J. Thiesen, Å. Glass, D. Winder, S. Liebe, J. Emmrich, Am. J. Physiol.: Gasterointest. Liver Physiol. 287 (2004) G211.
[22] H. Aoki, H. Ohnishi, K. Hama, T. Ishijima, Y. Satoh, K. Hanatsuka, A. Ohashi, S. Wada, T. Miyata, H. Kita, H. Yamamoto, H. Osawa, K. Sato, K. Tamada, H. Yasuda, H. Mashima, K. Sugano, Am. J. Physiol., Cell Physiol. 290 (2006) C1100.
[23] H. Aoki, H. Ohnishi, K. Hama, S. Shinozaki, H. Kita, H. Yamamoto, H. Osawa, K. Sato, K. Tamada, K. Sugano, J. Cell. Biochem. 99 (2006) 221.
[24] B. Fitzner, P. Brock, H. Nechutova, A. Glass, T. Karopka, D. Koczan, H.J. Thiesen, G. Sparmann, J. Emmrich, S. Liebe, R. Jaster, Cell. Signal. 19 (2007) 782.
[25] R. Jaster, J. Emmrich, Best. Pract. Res. Clin. Gastroenterol. 22 (2008) 17.
[26] M.J. DiMagno, E.P. Dimagno, Curr. Opin. Gastroenterol. 22 (2006) 487.
[27] R. Talukdar, N. Saikia, D.K. Singal, R. Tandon, Pancreatology 6 (2006) 440.
[28] M.J. Arthur, J.P. Iredale, D.A. Mann, Alcohol Clin. Exp. Res. 23 (1999) 940.
[29] N. Ohnishi, T. Miyata, H. Ohnishi, H. Yasuda, K. Tamada, N. Ueda, H. Mashima, K. Sugano, Gut 52 (2003) 1487.
[30] E. Schneider, A. Schmid-Kotsas, J. Zhao, H. Weidenbach, R.M. Schmid, A. Menke, G. Adler, J. Waltenberger, A. Grünert, M.G. Bachem, Am. J. Physiol., Cell Physiol. 281 (2001) C532.
[31] D.J. Abraham, X. Shiwen, C.M. Black, S. Sa, Y. Xu, A. Leask, J. Biol. Chem. 275 (2000) 15220.
[32] L.A. Cooker, D. Peterson, J. Rambow, M.L. Riser, R.E. Riser, F. Najmabadi, D. Brigstock, B.L. Riser, Am. J. Physiol. Renal. Physiol. 293 (2007) F157.